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(21) International Application Number: PCT/US97/17029 (22) International Filing Date: 23 September 1997 (23.09.97) (30) Priority Data: 60/027,274 27 September 1996 (27.09.96) US 9625802.5 12 December 1996 (12.12.96) GB (71) Applicant (for all designated States except US): MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SARDANA, Vinod, V. [IN/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). BLUE, Jeffrey, T. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). (74) Common Representative: MERCK & CO., INC.; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).		(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, HU, ID, IL, IS, JP, KG, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: DETERGENT-FREE HEPATITIS C PROTEASE (57) Abstract The protease of Hepatitis C virus (HCV) is purified without detergent, and is useful as a screening tool for HCV antivirals as well as a diagnostic tool for diseases resulting from HCV infection.		

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EE	Estonia						

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TITLE OF THE INVENTION
DETERGENT-FREE HEPATITIS C PROTEASE

BACKGROUND OF THE INVENTION

Hepatitis C virus (HCV) infection is found in 0.5% to 8.0% of blood donors worldwide. Because the infection is chronic in more than 60% of infected persons, the disease is an important public health and economic problem. The management of patients with chronic hepatitis C is complex—the disease is often only mildly symptomatic and slowly progressive, but 20% of patients develop cirrhosis after 20 years of infection and perhaps 10% of those with cirrhosis develop hepatocellular carcinoma. It is also an important indication for liver transplantation. In Europe and Japan the disease is more important numerically than is either hepatitis B or HIV infection. Existing antiviral agents are effective in only a minority of patients, yet good responses can be obtained.

An important target for the treatment of HCV is nonstructural protein 3, a protease encoded by HCV. This NS3 protease associated with human hepatitis C virus is an unstable protein in the absence of high concentrations of detergent. To stabilize the NS3 protease to sufficient quantities for biochemical, kinetic, and biophysical analyses, as well as for the construction of antiviral screening assays, ionic or non-ionic detergents need be incorporated both during purification and analyses. Antiviral leads discovered with detergent treated NS3 protein are not useful. Further, the presence of high quantities of detergents renders significant difficulties in the precise interpretations of biochemical, kinetic, and biophysical analyses. In some cases (e.g., sedimentation, protein crystallization), the presence of detergents preclude biochemical, kinetic, and biophysical analyses.

Prior methods employed detergents and glycerol for purification of NS3. Applicants have discovered a method of purifying NS3 without detergent, with from 5% to about 20% glycerol, preferably 7-12% and with high stability and activity. The resultant enzyme displays a higher catalytic activity than what is known for this

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protease, that is, 10-500 fold more active than the prior art preparations, depending on the form of the enzyme. Purification according to the methods of the present invention ensures a high stability of the NS3 protease, rendering it amenable to kinetic, biochemical, and biophysical analyses in the absence of detergents. Prior art methods do not afford a stable, detergent free NS3 for enzymologic, biochemical, and biophysical studies.

When properly expressed and prepared from the cloned plasmid in *E. coli*, the NS3 protease is obtained in milligram quantities in the complete absence of detergent. The resultant enzyme is very soluble and stable for long periods of time (weeks to months at 4°C and >12 months at -80°C), and displays high catalytic activity.

An assay with the detergent free HCV NS3 protease is useful as a screening tool for HCV antivirals as well as a diagnostic tool for diseases resulting from HCV infection. The potency range of the HCV antivirals can range from subnanomolar to micromolar concentrations.

BRIEF DESCRIPTION OF THE INVENTION

Detergent free NS3 protease of Hepatitis C virus (HCV) is prepared, and a screening assay for the protein inhibitors is constructed. The detergent free NS3 protease is useful as a screening tool for HCV antivirals, as well as a diagnostic tool for diseases resulting from HCV infection.

DETAILED DESCRIPTION OF THE INVENTION

ABBREVIATIONS AND DEFINITIONS

HCV	Hepatitis C Virus
IPTG	Isopropyl-D(-)thiogalactopyranoside
NS3	Nonstructural protein 3 of Hepatitis C virus

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PMSF	Phenylmethylsulfonyl fluoride
EDTA	ethylenediamino-tetraacetic acid
DTT	dithiothreitol
r.p.m.	Revolutions per minute
PAGE	Polyacrylamide gel
SDS	Sodium dodecylsulfate
NS	non-structural
HPLC	High Performance Liquid Chromatography

In one aspect of the invention a stable, detergent free HCV NS3 protease is claimed.

In another aspect of the invention a screening assay for the detection of compounds that inhibit HCV NS3 protease is claimed.

In still another aspect of the invention, the compounds that inhibit HCV NS3 protease as measured by the screening assay of Claim 2 are claimed.

In yet another aspect of the invention, a process for purifying active HCV NS3 protease without detergent is claimed.

There is disclosed stable, detergent free nonstructural protein 3 of Hepatitis C virus, also known as NS3 protease of HCV or NS3. The NS3 protease is useful screening tool for HCV antivirals, as well as a diagnostic tool for diseases resulting from HCV infection.

One utility for HCV NS3 protease is a screening assay for the detection of compounds that inhibit HCV NS3 protease. This assay has a procedure comprising the steps of:

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- (a) providing a quantity of a compound or compounds to be assayed;
- (b) incubating said compound or compounds with detergent free HCV NS3 protease in an HCV NS3 protease assay;
- (c) determining the inhibition of said protease in the HCV NS3 protease substrate cleavage assay.

Also encompassed in the present invention are compounds that substantially inhibit the HCV NS3 protease.

This invention also relates to a process for purifying active HCV NS3 protease without detergent and with from 5% to about 20% glycerol, comprising the steps of:

- (a) providing a quantity of cells expressing HCV NS3 protease;
- (b) disrupting the cells to form a suspension in buffer without detergent;
- (c) centrifuging the suspension to remove particulate matter;
- (d) subjecting the supernatant of step (c) to one or more steps of ion exchange chromatography under eluting buffer conditions without detergent;
- (e) to give active HCV NS3 protease in buffer without detergent.

One embodiment of the process for purifying active HCV NS3 protease without detergent and with from 7% to about 12% glycerol, comprises the steps of:

- (a) providing a quantity of cells expressing HCV NS3 protease;

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- (b) disrupting the cells with a microfluidizer to form a suspension in buffer without detergent, said buffer having pH of between about 6.5 and about 7.5;
- (c) centrifuging the suspension to remove particulate matter, at between about 5000 and about 8000 r.p.m. for about 15 minutes;
- (d) subjecting the supernatant of step (c) to one or more steps of cation exchange chromatography under eluting buffer conditions in a salt or pH gradient without detergent;
- (e) to give active HCV NS3 protease in buffer without detergent.

The nonstructural protein 3 of Hepatitis C virus, also known as NS3 protease, can exist in active form as an enzyme or as a complex with the cofactor . It has been discovered by applicants that the complex is about 1000 times more active than the enzyme by itself. The enzyme is itself about 10 times more active than prior art preparations purified with detergent. In the screening assays of the present invention, all active forms are encompassed.

Expression of HCV NS3 Protease in a Recombinant Expression System

It is now a relatively straightforward technology to prepare cells expressing a foreign gene. Such cells act as hosts and include *E. coli*, *B. subtilis*, yeasts, fungi, plant cells or animal cells. Expression vectors for many of these host cells have been isolated and characterized, and are used as starting materials in the construction, through conventional recombinant DNA techniques, of vectors having a foreign DNA insert of interest. Any DNA is foreign if it does not naturally derive from the host cells used to express the DNA insert. The foreign DNA insert may be expressed on extrachromosomal plasmids or after integration in whole or in part in the host cell chromosome(s), or may actually exist in the host cell as a combination of more than one molecular form. The choice of host cell and

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expression vector for the expression of a desired foreign DNA largely depends on availability of the host cell and how fastidious it is, whether the host cell will support the replication of the expression vector, and other factors readily appreciated by those of ordinary skill in the art.

The technology for recombinant procaryotic expression systems is now old and conventional. The typical host cell is *E. coli*. The technology is illustrated by treatises such as Wu, R (ed) *Meth. Enzymol.*, 68 (1979) and Maniatis, T. *et al.*, *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor 1982.

The foreign DNA insert of interest comprises a DNA sequence coding for HCV NS3 protease (or stable functional mutant thereof) of the present invention, including any synthetic sequence with this coding capacity or any such cloned sequence or combination thereof. For example, HCV peptides coded and expressed by an entirely recombinant DNA sequence is encompassed by this invention.

Vectors useful for constructing eukaryotic expression systems for the production of recombinant HCV comprise the DNA sequence for HCV or variant thereof, operatively linked thereto with appropriate transcriptional activation DNA sequences, such as a promoter and/or operator. Other typical features may include appropriate ribosome binding sites, termination codons, enhancers, terminators, or replicon elements. These additional features can be inserted into the vector at the appropriate site or sites by conventional splicing techniques such as restriction endonuclease digestion and ligation.

Yeast expression systems, which are one variety of recombinant eukaryotic expression systems, generally employ *Saccharomyces cerevisiae* as the species of choice for expressing recombinant proteins. *S. cerevisiae* and similar yeasts possess well known promoters useful in the construction of yeast expression systems, including but not limited to GAP491, GAL10, ADH2, and alpha mating factor.

Yeast vectors useful for constructing recombinant yeast expression systems for expressing HCMV include, but are not limited to,

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shuttle vectors, cosmids, chimeric plasmids, and those having sequences derived from 2-micron circle plasmids.

Insertion of the appropriate DNA sequence coding for HCV, into these vectors will, in principle, result in a useful recombinant yeast expression system for HCV where the modified vector is inserted into the appropriate host cell, by transformation or other means.

One preferred expression system is with baculovirus, under the control of the polyhedrin promoter or the p10 promoter. See, e.g., D.R. O'Reilly *et al.*, *Baculovirus Expression Vectors: A Laboratory Manual* W.H. Freeman 1992, for a background description of this expression technology. This system employs the isolation of a recombinant baculovirus carrying the gene of interest. The baculovirus system is especially useful for the simultaneous expression of more than one protein.

Recombinant mammalian expression systems are another means of producing the recombinant HCV for the conjugates of this invention. In general, a host mammalian cell can be any cell that has been efficiently cloned in cell culture. Host mammalian cells useful for the purposes of constructing a recombinant mammalian expression system include, but are not limited to, Vero cells, NIH3T3, GH3, COS, murine C127 or mouse L cells. Mammalian expression vectors can be based on virus vectors, plasmid vectors which may have SV40, BPV or other viral replicons, or vectors without a replicon for animal cells. Detailed discussions on mammalian expression vectors can be found in the treatises of Glover, D.M. (ed.) "DNA Cloning: A Practical Approach," IRL 1985, *Vols. I and II*.

Recombinant HCV may possess additional and desirable structural modifications not shared with the same organically synthesized peptide, such as adenylation, carboxylation, glycosylation, hydroxylation, methylation, phosphorylation, myristoylation, extension or trimming of either the amino- or carboxy-terminal ends or both. These added features may be chosen or preferred as the case may be, by the appropriate choice of recombinant expression system. On the other

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hand, recombinant HCV may have its sequence extended by the principles and practice of organic synthesis.

Purification

In the detergent free purification process of the present invention, virtually any source of NS3 protease is suitable, whether recombinant or not. Preferred sources are recombinant, most preferred is an expression system using *E.coli*.

For expression systems, whether or not recombinant, the NS3 must first be isolated in a soluble fraction. Cells expressing HCV protease are disrupted in buffer to form a suspension in buffer. The disruption is carried out by any of a variety of well known techniques, including but not limited to treatment with a French press, a microfluidizer, sonicator, or self-digestion by inductive expression of lysozyme. A preferred technique of disruption is with a microfluidizer.

Throughout the purification process without detergent, it is critical to maintain the pH of any buffer between about 6.5 and about 7.5.

The next step involves initial fractionation of the suspension of cellular debris. The suspension is treated to separate the soluble fraction from particulate matter, or such other step is performed that substantially separates soluble from insoluble protein. Appropriate techniques include, but are not limited to, centrifugation at between about 5,000 r.p.m. and about 8,000 r.p.m. for about 15 minutes, filtration, or salt precipitation with e.g. $(\text{NH}_4)_2\text{SO}_4$. It is understood that these initial fractionation procedures are well known and are subject to many variations. Appropriate modifications in the initial fractionation of NS3 are well within the skill of the art. The preferred method is centrifugation.

Initial fractionation of the suspension of cellular debris results in a supernatant as well as a precipitate or insoluble pellet. The supernatant is treated further.

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The supernatant is then subjected to one or more steps of ion exchange chromatography, and, optionally, gel filtration, to give a substantially pure NS3 in buffer without detergent. Preferred ion exchangers include, but are not limited to, cation exchangers on polystyrene, cation exchangers on dextran, cation exchangers on agarose, cation exchangers on cellulose, or heparin. The cation exchanger is typically a strongly or weakly acidic side chain residue. The ion exchanger is washed in a gradient of salt and/or pH to elute specifically NS3 protease. Preferred eluting conditions are a salt gradient.

Such ion exchange chromatography can be repeated or varied until substantially pure NS3 protease is obtained. Typically two rounds of cation exchange chromatography are employed.

Preferred storage conditions involve having the enzyme in 25mM HEPES (pH 7.5), 10% glycerol, 10mM DTT and approximately 300mM sodium chloride at a concentration of ~15uM or above at -80°C.

EXAMPLE 1

Expression of the HCV NS3 Protease

Plasmid DNA encoding amino acids 1027-1206 of the BK strain HCV polypeptide was cloned downstream of the T7-7 vector, in frame with the first ATG of the protein of gene 10 of the T7 phage, to obtain the plasmid pT7-7 (NS31027-1206), using methods that are known to the molecular biology practice. See PCT WO 95/22985, published August 31, 1995, incorporated by reference. This plasmid was transfected into *E. coli* BL21DE3 plysS cells (Novagen) utilizing the heat-shock technique. Cells were grown at 37°C in LB medium containing 50 ug/ml ampicillin to an optical density of 0.4-0.6 at 600 nm whereupon the temperature was lowered to 25°C and expression of NS3 was induced with 400 uM IPTG. Cells were allowed to grow further for two hours and then harvested by centrifugation and stored at -80°C until lysis.

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EXAMPLE 2Purification of the HCV NS3 Protease in the Absence of Detergents

Cells from a 10-L culture were re-suspended in 100 ml of lysis buffer (25 mM sodium phosphate pH 7.5, 1 mM EDTA, 10% glycerol, 5 mM DTT) at 4°C and treated with 0.02 mg/ml DNase (Type IIS: Bovine Pancreas Sigma) in 20 mM MgCl₂ for 30 min. PMSF (1 mM) was added to the suspension and cells were immediately disrupted by placing them 6 times through a microfluidizer at a pressure of 6 Bar. The lysate was centrifuged at 10,000 rpm for 30 min, and the supernatant was collected and loaded onto a cation exchange column (Hi-Load SP Sepharose High Performance) pre-equilibrated in 50 mM sodium phosphate pH 6.5, 10% glycerol, 1 mM EDTA, 5 mM DTT, at a flow rate of 2.5 ml/min. The NS3 protease was eluted from the column in a 0-1 M NaCl salt gradient. Fractions were analyzed by SDS-PAGE. Fractions containing the NS3 protease were pooled and first diluted 8-10 fold into a buffer containing 25 mM sodium phosphate (pH 7.5), 10% glycerol, 5 mM DTT buffer and then loaded onto two 4x5 ml Heparin columns connected in tandem at a flow rate of 3 ml/min. The enzyme was eluted with a NaCl gradient. Fractions were analyzed by SDS-PAGE and peptide cleavage assay. Enzyme fractions containing > 95% pure NS3 protease were pooled and stored at 4°C in the elution buffer. The yield was 1-2 mg of purified enzyme per liter of *E. Coli* cell culture. N-terminal sequence analyses were carried out using the Edman degradation method using an Applied Biosystem model 470A gas phase sequencer. The protease concentration was determined by quantitative amino acid analysis.

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EXAMPLE 3HCV NS3 Substrate Cleavage Assay

The peptides (7-methoxycoumarin-4-acetyl-DEMEECASHLPYK-(ϵ -NHCOCH₃) and acetyl-DEMEECASHLPYK-(ϵ -NHCOCH₃) mimicking the NS4A/4B cleavage site was purchased from Enzyme Systems Products (Dublin, CA) and was > 95% pure. A lysine was added to the C-terminus of the acetyl-DEMEECASHLPYK-(ϵ -NHCOCH₃) peptide to enable it soluble at high concentrations and a coumarin fluorophore was introduced to the N-terminus of the (7-methoxycoumarin-4-acetyl-DEMEECASHLPYK-(ϵ -NHCOCH₃) peptide to enhance detection of the product. The NS4B/5A substrate 7-methoxycoumarin-4-acetyl-EDASTPCSGS-Nph-L (where Nph = para-nitro phenylalanine) was purchased from Bachem Biosciences. The 4A peptide with the sequence of GSVVIVGRIILSGRKK was also synthesized by Enzyme System Products. Peptide cleavage assays were conducted at 25°C in 100 μ l of 50 mM Hepes (pH 7.5) reaction buffer, 10 mM DTT in the presence of varying amounts of glycerol, preferably 0% to 50% glycerol. The reaction was quenched with 100 μ l of 5% phosphoric acid and the mixture was analyzed by reverse phase HPLC on a 4.6/50 mm Vydac C18 column. The cleavage products were separated using a 0.1% phosphoric acid/acetonitrile gradient and identified by comparison of retention time with authentic peptides representing the reaction products. Cleavage of the NS4A/4B occurred at the expected Cys-Ala scissile bond. UV Absorbance of the products was monitored at 220 nm and fluorescence detection done with with excitation and emission wavelengths set at 328 nm and 393 nm, respectively. The enzyme concentrations used in the assays varied from, but not limiting to, 2 to 1000 nM depending on the reaction conditions desired. For example, the enzyme concentration in the presence of the 4A peptide varied from 2 to 50 nM and 300 to 1000 nM in the absence of the 4A peptide. In the assays with the 4A peptide as the cofactor, the enzyme was preincubated at a temperature of about 0°C to 10°C with the 4A peptide for 5 to 10 minutes, followed by 3 to 10 minutes at room

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temperature at a 10-50 fold greater concentration, before the onset of reaction. For preincubation of enzyme with the 4A peptide, the enzyme was added to the solution already containing the 4A peptide. The concentration of 4A peptide and substrate used ranged from, but not limiting to, 75 nM to 50 μ M and 0.1 μ M to 250 μ M, respectively. All substrates were dissolved in 50 mM HEPES (pH 7.5), 30 mM DTT and 10% glycerol. The reaction was typically allowed to continue for a period of 2.5 to 15 min depending on the initial reaction rate and the sensitive detection of products.

Steady state kinetic parameters (k_{cat} and K_M) were determined by fitting initial rates (obtained at <5% of total substrate hydrolyzed) verses substrate concentrations to the Michaelis-Menten equation. Initial velocity and steady-state conditions were strictly maintained for all reaction assays performed.

While the foregoing specification teaches the principles of the present invention, with examples provided for the purpose of illustration, it will be understood that the practice of the invention encompasses all of the usual variations, adaptations, modifications, deletions or additions of procedures and protocols described herein, as come within the scope of the following claims and its equivalents.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Sardana, Vinod V
Blue, Jeffrey T
- (ii) TITLE OF INVENTION: DETERGENT-FREE HEPATITIS C PROTEASE
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: MERCK & CO., INC.
 - (B) STREET: P.O. Box 2000, 126 E. Lincoln Ave.
 - (C) CITY: Rahway
 - (D) STATE: NJ
 - (E) COUNTRY: US
 - (F) ZIP: 07065-0907
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Ayler, Sylvia A
 - (B) REGISTRATION NUMBER: 36,436
 - (C) REFERENCE/DOCKET NUMBER: 19691PV
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 908-594-4909
 - (B) TELEFAX: 908-594-4720

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 631 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (v) FRAGMENT TYPE: internal

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(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Hepatitis C. Virus
 (B) STRAIN: NS3 Serine Protease Domain
 (C) INDIVIDUAL ISOLATE: BK

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: described by Tomei et al. in 1993
 (B) CLONE: cDNA clone pCD (38-9.4)

(viii) POSITION IN GENOME:

- (B) MAP POSITION: 1-180

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ala	Pro	Ile	Thr	Ala	Tyr	Ser	Gln	Gln	Thr	Arg	Gly	Leu	Leu	Gly	Cys	1	5	10	15
Ile	Ile	Thr	Ser	Leu	Thr	Gly	Arg	Asp	Lys	Asn	Gln	Val	Glu	Gly	Glu	20	25	30	
Val	Gln	Val	Val	Ser	Thr	Ala	Thr	Gln	Ser	Phe	Leu	Ala	Thr	Cys	Val	35	40	45	
Asn	Gly	Val	Cys	Trp	Thr	Val	Tyr	His	Gly	Ala	Gly	Ser	Lys	Thr	Leu	50	55	60	
Ala	Gly	Pro	Lys	Gly	Pro	Ile	Thr	Gln	Met	Tyr	Thr	Asn	Val	Asp	Gln	65	70	75	80
Asp	Leu	Val	Gly	Trp	Gln	Ala	Pro	Pro	Gly	Ala	Arg	Ser	Leu	Thr	Pro	85	90	95	
Cys	Thr	Cys	Gly	Ser	Ser	Asp	Leu	Tyr	Leu	Val	Thr	Arg	His	Ala	Asp	100	105	110	
Val	Ile	Pro	Val	Arg	Arg	Arg	Gly	Asp	Ser	Arg	Gly	Ser	Leu	Leu	Ser	115	120	125	
Pro	Arg	Pro	Val	Ser	Tyr	Leu	Lys	Gly	Ser	Ser	Gly	Gly	Pro	Leu	Leu	130	135	140	
Cys	Pro	Ser	Gly	His	Ala	Val	Gly	Ile	Phe	Arg	Ala	Ala	Val	Cys	Thr	145	150	155	160
Arg	Gly	Val	Ala	Lys	Ala	Val	Asp	Phe	Val	Pro	Val	Glu	Ser	Met	Glu	165	170	175	
Thr	Thr	Met	Arg	Ser	Pro	Val	Phe	Thr	Asp	Asn	Ser	Ser	Pro	Pro	Ala	180	185	190	
Val	Pro	Gln	Ser	Phe	Gln	Val	Ala	His	Leu	His	Ala	Pro	Thr	Gly	Ser	195	200	205	

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Gly Lys Ser Thr Lys Val Pro Ala Ala Tyr Ala Ala Gln Gly Tyr Lys
 210 215 220
 Val Leu Val Leu Asn Pro Ser Val Ala Ala Thr Leu Gly Phe Gly Ala
 225 230 235 240
 Tyr Met Ser Lys Ala His Gly Ile Asp Pro Asn Ile Arg Thr Gly Val
 245 250 255
 Arg Thr Ile Thr Thr Gly Ala Pro Val Thr Tyr Ser Thr Tyr Gly Lys
 260 265 270
 Phe Leu Ala Asp Gly Gly Cys Ser Gly Gly Ala Tyr Asp Ile Ile Ile
 275 280 285
 Cys Asp Glu Cys His Ser Thr Asp Ser Thr Thr Ile Leu Gly Ile Gly
 290 295 300
 Thr Val Leu Asp Gln Ala Glu Thr Ala Gly Ala Arg Leu Val Val Leu
 305 310 315 320
 Ala Thr Ala Thr Pro Pro Gly Ser Val Thr Val Pro His Pro Asn Ile
 325 330 335
 Glu Glu Val Ala Leu Ser Asn Thr Gly Glu Ile Pro Phe Tyr Gly Lys
 340 345 350
 Ala Ile Pro Ile Glu Ala Ile Arg Gly Gly Arg His Leu Ile Phe Cys
 355 360 365
 His Ser Lys Lys Lys Cys Asp Glu Leu Ala Ala Lys Leu Ser Gly Leu
 370 375 380
 Gly Ile Asn Ala Val Ala Tyr Tyr Arg Gly Leu Asp Val Ser Val Ile
 385 390 395 400
 Pro Thr Ile Gly Asp Val Val Val Val Ala Thr Asp Ala Leu Met Thr
 405 410 415
 Gly Tyr Thr Gly Asp Phe Asp Ser Val Ile Asp Cys Asn Thr Cys Val
 420 425 430
 Thr Gln Thr Val Asp Phe Ser Leu Asp Pro Thr Phe Thr Ile Glu Thr
 435 440 445
 Thr Thr Val Pro Gln Asp Ala Val Ser Arg Ser Gln Arg Arg Gly Arg
 450 455 460
 Thr Gly Arg Gly Arg Arg Gly Ile Tyr Arg Phe Val Thr Pro Gly Glu
 465 470 475 480
 Arg Pro Ser Gly Met Phe Asp Ser Ser Val Leu Cys Glu Cys Tyr Asp
 485 490 495
 Ala Gly Cys Ala Trp Tyr Glu Leu Thr Pro Ala Glu Thr Ser Val Arg
 500 505 510

- 16 -

Leu Arg Ala Tyr Leu Asn Thr Pro Gly Leu Pro Val Cys Gln Asp His
 515 520 525
 Leu Glu Phe Trp Glu Ser Val Phe Thr Gly Leu Thr His Ile Asp Ala
 530 535 540
 His Phe Leu Ser Gln Thr Lys Gln Ala Gly Asp Asn Phe Pro Tyr Leu
 545 550 555 560
 Val Ala Tyr Gln Ala Thr Val Cys Ala Arg Ala Gln Ala Pro Pro Pro
 565 570 575
 Ser Trp Asp Gln Met Trp Lys Cys Leu Ile Arg Leu Lys Pro Thr Leu
 580 585 590
 His Gly Pro Thr Pro Leu Leu Tyr Arg Leu Gly Ala Val Gln Asn Glu
 595 600 605
 Val Thr Leu Thr His Pro Ile Thr Lys Tyr Ile Met Ala Cys Met Ser
 610 615 620
 Ala Asp Leu Glu Val Val Thr
 625 630

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: cDNA clone (See Seq. ID No:1)
- (B) CLONE: NS4A Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Thr Trp Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala Tyr
 1 5 10 15
 Cys Leu Thr Thr Gly Ser Val Val Ile Val Gly Arg Ile Ile Leu Ser
 20 25 30

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Gly Arg Pro Ala Ile Val Pro Asp Arg Glu Leu Leu Tyr Gln Glu Phe
 35 40 45

Asp Glu Met Glu Glu Cys
 50

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Cofactor of NS3 serine protease
- (B) CLONE: Solid phase peptide synthesis

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Ser Val Val Ile Val Gly Arg Ile Ile Leu Ser Gly Arg Pro Ala
 1 5 10 15

Ile Val Pro Asp Arg Glu Val Leu Tyr Gln Glu Phe Asp Glu Met Glu
 20 25 30

Glu Asx

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WHAT IS CLAIMED IS:

1. Stable, detergent free HCV NS3 protease.
2. A screening assay for the detection of compounds that inhibit HCV NS3 protease, said assay having a procedure comprising the steps of:
 - (a) providing a quantity of a compound or compounds to be assayed;
 - (b) incubating said compound or compounds with the HCV NS3 protease of Claim 1 in an HCV NS3 protease assay;
 - (c) determining the inhibition of said protease in the HCV NS3 protease assay.
3. The compounds that inhibit HCV NS3 protease as measured by the screening assay of Claim 2.
4. A process for purifying active HCV NS3 protease without detergent, comprising the steps of:
 - (a) providing a quantity of cells expressing HCV NS3 protease;
 - (b) disrupting the cells to form a suspension in buffer without detergent;
 - (c) centrifuging the suspension to remove particulate matter;
 - (d) subjecting the supernatant of step (c) to one or more steps of ion exchange chromatography under eluting buffer conditions without detergent;
 - (e) to give active HCV NS3 protease in buffer without detergent.

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5. A process for purifying active HCV NS3 protease without detergent, comprising the steps of:

- (a) providing a quantity of cells expressing HCV NS3 protease;
- (b) disrupting the cells with a microfluidizer to form a suspension in buffer without detergent, said buffer having pH of between about 6.5 and about 7.5;
- (c) centrifuging the suspension to remove particulate matter, at between about 5000 and about 8000 r.p.m. for about 15 minutes;
- (d) subjecting the supernatant of step (c) to one or more steps of cation exchange chromatography under eluting buffer conditions in a salt or pH gradient without detergent;
- (e) to give active HCV NS3 protease in buffer without detergent.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17029

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 9/48, 9/50, 9/96

US CL : 435/188, 212, 219

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/188,212,219

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHOJI et al. Protelytic Activity of NS3 Serine Proteinase of Hepatitis C Virus Efficiently Expressed in Escherichia coli. Hepatology. December 1995, Volume 22, Number 6, pages 1648-1655, see page 1648, abstract, page 1649, column 2, third paragraph, and page 1652, column 1-2, second full paragraph.	1-5
Y	D'SOUZA et al. In vitro Cleavage of Hepatitis C Virus Polypeptide Substrates by Purified Recombinant NS3 Protease. Journal of General Virology. July 1995, Volume 76, pages 1729-1736, see page 1730, column 1, paragraphs 4 and 6 and page 1733, columns 1-2, first full paragraph.	1-5

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 DECEMBER 1997

Date of mailing of the international search report

07 JAN 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

BRENDA BRUMBACK

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17029

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LIN et al. "The Hepatitis C Virus NS3 Serine Proteinase and NS4A Cofactor: Establishment of a Cell-Free Trans-Processing Assay. Proceedings of the National Academy of Science. August 1995, Volume 92, pages 7622-7626, see page 7625, column 1, third paragraph and page 7626, column 1, third paragraph.	1-5
Y	Wondrak et al., "Purification of HIV-1 Wild-Type Protease and Characterization of Proteolytically Inactive HIV-1 Protease Mutants by Pepstatin A Affinity Chromatography, FEBS Letters. March 1991, Volume 280, No. 2, pages 347-350, see page 347, column 2, first full paragraph.	1-5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17029

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/17029

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG: MEDLINE, BIOTECH, CONF PAPERS, EURO PATENTS, JAPIO, WPI
search terms: hepatitis c virus, HCV, protease, inhibit?, assay, purify?, isolat?, express?, glycerol

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I: Claims 1,2,4, and 5 drawn to HCV NS3 protease, a method of making, and a method of use.

Group II: Claim 3 drawn to compounds that inhibit HCV protease.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: They are drawn to different groups of compounds and are mutually exclusive.